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# The ability of a new hypoglycaemic agent, A-4166, compared to sulphonylureas, to increase cytosolic $Ca^{2+}$ in pancreatic $\beta$-cells under metabolic inhibition

*†Shoji Fujitani, †Kyoko Okazaki & [1,]*Toshihiko Yada

*Department of Physiology, Kagoshima University School of Medicine, Kagoshima 890 and †Life Science Laboratories, Central Research Laboratories, Ajinomoto Co. Inc., Yokohama 244, Japan

**1** *N*-(*trans*-4-isopropylcyclohexanecarbonyl)-D-phenylalanine (A-4166) is a new non-sulphonylurea oral hypoglycaemic agent which stimulates insulin release by increasing cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) in $\beta$-cells.

**2** We studied comparative effects of A-4166 and sulphonylureas on $[Ca^{2+}]_i$, measured by dual-wavelength fura-2 microfluorometry, in single rat pancreatic $\beta$-cells under normal conditions and conditions where glucose metabolism was inhibited.

**3** A glucokinase inhibitor, mannoheptulose (10 mM), a mitochondrial respiratory inhibitor, KCN (100 $\mu$M), and uncouplers, dinitrophenol (DNP, 50 $\mu$M) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 0.3 $\mu$M), were used to abolish glucose-induced increases in $[Ca^{2+}]_i$ in a reversible manner.

**4** Under control conditions, A-4166 was one order more potent than tolbutamide in increasing $[Ca^{2+}]_i$, and maximal responses were evoked by 30 $\mu$M A-4166 and 300 $\mu$M tolbutamide. These equipotent concentrations were employed for the comparative study where glucose metabolism was inhibited.

**5** In the presence of mannoheptulose, $[Ca^{2+}]_i$ responses to tolbutamide, but not those to A-4166, were attenuated in a reversible manner.

**6** KCN, DNP and FCCP inhibited $[Ca^{2+}]_i$ responses to tolbutamide to a much greater extent than those to A-4166. Responses to tolbutamide even at 3.3 times the equipotent concentration (1000 $\mu$M) were also markedly attenuated by these inhibitors. Responses evoked by another sulphonylurea, gliclazide, were inhibited by DNP to a larger extent than A-4166-induced responses.

**7** The results indicate that A-4166 acts more effectively than sulphonylureas to increase $[Ca^{2+}]_i$ in $\beta$-cells during metabolic inhibition.

**Keywords:** A-4166; sulphonylurea; metabolic inhibitor; pancreatic $\beta$-cell; cytosolic $Ca^{2+}$; NIDDM; glucose

## Introduction

*N*-(*trans*-4-isopropylcyclohexanecarbonyl)-D-phenylalanine (A-4166), a D-phenylalanine derivative, is a new non-sulphonylurea oral hypoglycaemic agent (Shinkai *et al*., 1988; 1989). The hypoglycaemic effect of A-4166 is mainly due to the stimulation of insulin release (Sato *et al*., 1991; Hirose *et al*., 1994; 1995; Fujitani *et al*., 1996). A-4166 exhibits quicker and shorter-lasting insulinotropic and hypoglycaemic effects than sulphonylureas in dogs (Sato *et al*., 1991). A-4166 induces an increase in $[Ca^{2+}]_i$ in rat pancreatic $\beta$-cells and it is tightly linked to the stimulated insulin release (Fujitani & Yada, 1994; Fujitani *et al*., 1996).

In pancreatic $\beta$-cells, glucose is metabolized via glycolysis, mitochondrial respiration and oxidative phosphorylation to produce adenosine 5′-triphosphate (ATP), the key signal that triggers a sequence of processes finally leading to insulin exocytosis (Ashcroft & Rorsman, 1989). Therefore, defects in glycolysis or mitochondria are expected to impair the glucose handling and ATP synthesis by $\beta$-cells, resulting in reduction of insulin release. Mutations of glucokinase, a key enzyme of glycolysis, have been demonstrated in the maturity-onset diabetes of the young (MODY), a form of non-insulin-dependent diabetes mellitus (NIDDM) (Froguel *et al*., 1992; Velho *et al*., 1992). Mutations on the mitochondrial gene are associated with maternally transmitted NIDDM (Ballinger *et al*., 1992; van den Ouweland *et al*., 1992; Reardon *et al*., 1992; Kadowaki *et al*., 1994). NIDDM patients with mutations in the glucokinase or mitochondrial gene are characterized by an impaired insulin secretory response to glucose (Froguel *et al*., 1992; Velho *et al*., 1992; Suzuki *et al*., 1994). The mice where the $\beta$-cell specific disruption of the glucokinase gene is produced exhibit a defective insulin secretory response to glucose and develop NIDDM (Terauchi *et al*., 1995). Thus, defects in $\beta$-cell metabolism, including those in glucokinase and mitochondria, appear to be characteristically associated with and may cause NIDDM. Therefore, it is of importance to characterize the effects of the hypoglycaemic agents on $\beta$-cells whose metabolism is impaired.

In the present study, we isolated single $\beta$-cells from Wistar rats and treated them with a glucokinase inhibitor, a mitochondrial respiratory inhibitor and uncouplers to produce the $\beta$-cells in which metabolism is suppressed, as a model of NIDDM $\beta$-cells. We then investigated the effects of A-4166, in comparison to sulphonylureas, on $[Ca^{2+}]_i$ in these $\beta$-cells.

## Methods

### *Preparation and selection of islet β-cells*

Islets of Langerhans were isolated from Wistar rats aged 8–15 weeks by collagenase digestion. Animals were anaesthetized by intraperitoneal injection of pentobarbitone, 80 mg $kg^{-1}$. The abdomen was opened, and 3 mg $ml^{-1}$ collagenase (type IV, Worthington Biochemical, New Jersey, U.S.A.) dissolved in 5 ml of 5 mM $Ca^{2+}$-containing Krebs Ringer bicarbonate

[1]Author for correspondence at: Department of Physiology, Kagoshima University School of Medicine, 8-35-1 Sakuragaoka, Kagoshima 890, Japan.

buffer (KRB) solution was injected into the common bile duct at the distal end after ligation of the duct proximal to the pancreas. The pancreas was dissected out and incubated at 37°C for 17 min. Islets were collected and immediately dispersed into single cells in $Ca^{2+}$-free KRB. The single cells were then plated on coverslips and cultured for 1–4 days at 37°C in a 95% air + 5% $CO_2$ atmosphere in Eagle's MEM containing 5.6 mM glucose supplemented with 10% foetal bovine serum (FBS), 100 $\mu$g ml$^{-1}$ streptomycin and 100 u ml$^{-1}$ penicillin. The cells in culture for 1–4 days responded to A-4166 and tolbutamide in a consistent manner. $\beta$-Cells were selected according to the previously described procedure (Yada *et al*., 1993). Briefly, data were taken from the cells with a relatively larger diameter of 12.5–17.5 $\mu$m, when observed on coverslips, and with $[Ca^{2+}]_i$ responses to 16.7 mM glucose, the properties of immunocytochemically-confirmed $\beta$-cells (Yada *et al*., 1994).

### *Measurements of $[Ca^{2+}]_i$*

$[Ca^{2+}]_i$ was measured by dual-wavelength fura-2 microfluorometry (Grynkiewicz *et al*., 1985) according to the method described previously (Yada *et al*., 1992). In brief, the single cells on coverslips were incubated with 1 $\mu$M fura-2/acetoxymethylester for 30 min at 37°C in KRB containing 2.8 mM glucose. The cells were then mounted in a chamber, placed on the stage of an inverted TMD microscope (Nikon, Tokyo, Japan), and superfused at 1 ml min$^{-1}$ at 37°C with KRB containing either a basal (2.8 mM) or an elevated glucose concentration (16.7 mM). Superfusion was performed with a peristaltic pump (Watson Marlow, Cornwall, U.K.). Fura-2 loaded cells were alternately excited at 340 and 380 nm once per second through a 40x fluorite objective, the emission signals at 510 nm (F340 and F380) were detected by a photomultiplier, and the ratio of these signals (R = F340/F380) was recorded by a computer with a P1 microfluorometry system (Nikon). The ratio values were converted to $[Ca^{2+}]_i$ according to the calibration curve (Yada *et al*., 1992).

### *Normalization of amplitudes and classification of $[Ca^{2+}]_i$ responses*

To standardize the inhibition of $[Ca^{2+}]_i$ responses to A-4166 and sulphonylureas, the following procedures were employed. In each individual $\beta$-cell, the amplitude of the $[Ca^{2+}]_i$ response in the control condition at 2.8 mM glucose without inhibitors was normalized to 100% and the amplitude of the $[Ca^{2+}]_i$ response in a test condition was expressed as a percentage relative to the control. The normalized amplitude of $[Ca^{2+}]_i$ response (%) in each cell was averaged for all cells examined under a test condition to obtain the mean amplitude.

Under each test condition, the extent of inhibition of the $[Ca^{2+}]_i$ response to each stimulus varied between individual cells. The variation of inhibition by individual cells was evaluated by the following procedure: the normalized amplitude of $[Ca^{2+}]_i$ response was classified into 3 grades ($\leqslant$ 33.3%, 33.3–66.7% and $>$ 66.7%) and the distribution of individual cells between the 3 grades was analysed.

### *Solutions and chemicals*

Measurements were carried out in KRB solution composed of (in mM): NaCl 129, $NaHCO_3$ 5.0, KCl 4.7, $KH_2PO_4$ 1.2, $CaCl_2$ 1.0, $MgSO_4$ 1.2 and 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) 10 at pH 7.4 supplemented with 0.1% bovine serum albumin (BSA). FBS was obtained from Gibco (New York, U.S.A.). Fura-2 free acid, fura-2/acetoxymethylester and [ethylenebis-(oxyethylenenitrilo)] tetraacetic acid (EGTA) were purchased from Dojin Chemicals (Kumamoto, Japan). BSA was from Böehringer-Mannheim (Mannheim, Germany). A-4166 and gliclazide were synthesized at the Central Research Laboratories of Ajinomoto Inc. (Kanagawa, Japan). Tolbutamide, mannoheptulose, dinitrophenol (DNP), carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and potassium cyanide (KCN) were from Sigma (St. Louis, MO, U.S.A.).

### *Statistical analysis*

The calculated values are expressed as mean ± s.e.mean ($n$ = number of observations). Student's $t$ test was used for statistical analysis.

## Results

### *Effects of increasing concentrations of A-4166 and tolbutamide on $[Ca^{2+}]_i$ and determination of their equipotent concentrations*

The effects of A-4166 on $[Ca^{2+}]_i$ in rat pancreatic $\beta$-cells under control conditions at the basal glucose concentration of 2.8 mM were investigated and compared with those of tolbutamide. A-4166 at 3 $\mu$M evoked an increase in $[Ca^{2+}]_i$ in about 30%, at 10 $\mu$M in about 50% and at 30 $\mu$M or higher in all of the single $\beta$-cells examined, exhibiting a concentration-dependent effect. The $[Ca^{2+}]_i$ response to A-4166 usually appeared in a sustained pattern, while it was occasionally oscillatory (Figure 1a and 6a). A-4166 at 1 $\mu$M or less had no effect on $[Ca^{2+}]_i$ (Figure 2). Tolbutamide also concentration-dependently increased $[Ca^{2+}]_i$ in a sustained (Figure 1b) or some-

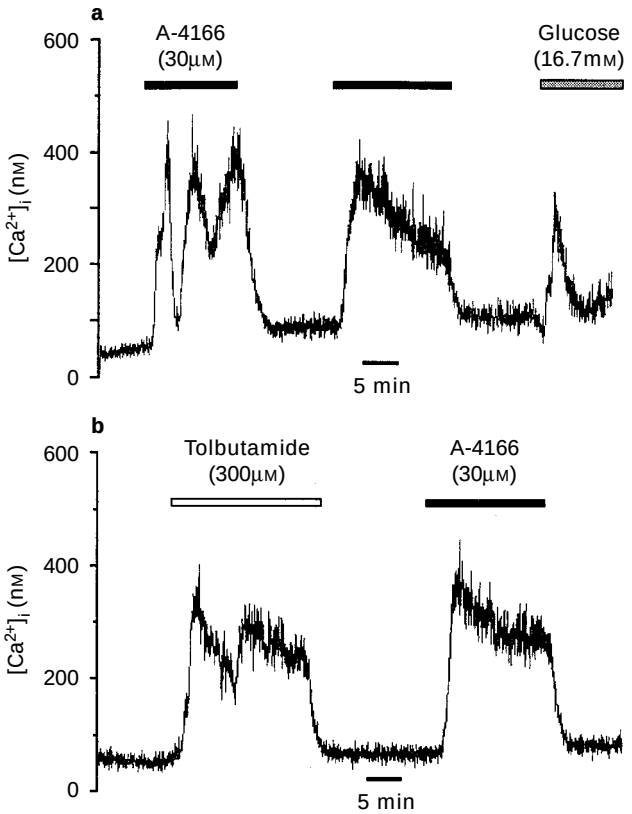


**Figure 1** Effects of A-4166, tolbutamide and 16.7 mM glucose on $[Ca^{2+}]_i$ in single rat pancreatic $\beta$-cells. (a) A-4166 30 $\mu$M was administered to a single $\beta$-cell superfused at 37°C with KRB containing 2.8 mM glucose and 0.1% BSA. Following stimulation with A-4166 twice, the glucose concentration was elevated to 16.7 mM. A-4166 evoked a rapid increase in $[Ca^{2+}]_i$ in a sustained or oscillatory pattern, while high glucose induced a bimodal change, an initial decrease and subsequent increase in $[Ca^{2+}]_i$. (b) A-4166 30 $\mu$M and tolbutamide 300 $\mu$M evoked sustained increases in $[Ca^{2+}]_i$ with similar amplitudes in the same single $\beta$-cell. $[Ca^{2+}]_i$ was calculated from the ratio of fura-2 fluorescence signals (F340/F380). Bars above the traces indicate periods of exposure to agents specified. Similar results were obtained in 25 cells in (a), and in 8 cells in (b).

times oscillatory pattern (Figure 4b). A-4166 and tolbutamide evoked a rapid increase in $[Ca^{2+}]_i$, whereas the elevation of the glucose concentration to 16.7 mM resulted in an initial decrease followed by a subsequent increase in $[Ca^{2+}]_i$, the bimodal response characteristic of the metabolized secretagogues (Gylfe, 1988; Yada *et al*., 1992). This result supports the previous finding that A-4166 as well as tolbutamide does not change β-cell metabolism (Fujitani & Yada, 1994). The amplitudes of A-4166- and tolbutamide-induced responses were nearly the same. The threshold and maximal concentrations of A-4166 capable of evoking an increase in $[Ca^{2+}]_i$ appeared to be 1–3 μM and 30 μM, while those of tolbutamide were 10–30 μM and 300 μM, respectively (Figure 2). Thus, at 2.8 mM glucose, A-4166 was as effective as, and its potency exactly one order greater than, tolbutamide (Figure 2). Accordingly, the following experiments were carried out with equipotent concentrations, 30 μM for A-4166 and 300 μM for tolbutamide, at which the two agents exert almost identical maximal effects. In some experiments, a supermaximal concentration of tolbutamide (1000 μM) was used to examine whether depression of the tolbutamide response by metabolic suppression could be relieved by increasing the concentration.

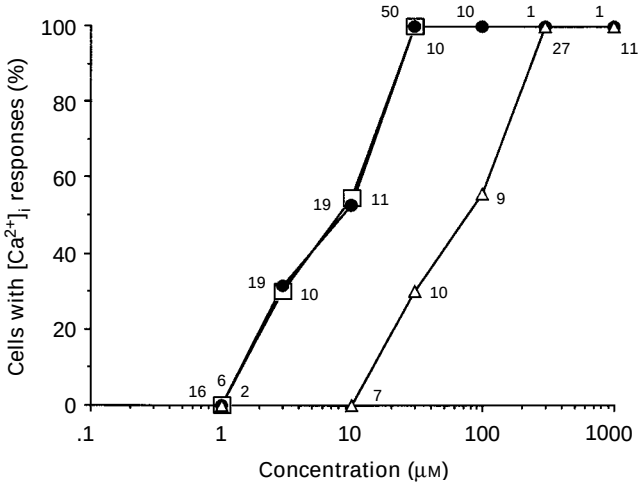


**Figure 2** Concentration-response characteristics for A-4166, tolbutamide and gliclazide to increase $[Ca^{2+}]_i$ in single rat pancreatic β-cells. The frequency, expressed as a percentage, of β-cells with $[Ca^{2+}]_i$ responses to various concentrations of (●) A-4166; (△) tolbutamide and (□) gliclazide. Glucose and BSA concentrations were 2.8 mM and 0.1%, respectively. Numbers near each symbol indicate the number of cells examined. When symbols overlap, the number on the left refers to A-4166, that on the right to gliclazide or tolbutamide, and that in the centre gliclazide.

### *Effects of inhibitors of glucokinase, mitochondrial respiratory chain and oxidative phosphorylation on glucose-induced increases in $[Ca^{2+}]_i$*

In the presence of 10 mM D-mannoheptulose, an inhibitor of glucose phosphorylation by glucokinase (Malaisse *et al*., 1968), a rise in glucose concentration from 2.8 mM to 16.7 mM failed to increase $[Ca^{2+}]_i$ in single β-cells (Figure 3a). After washing-out mannoheptulose, 16.7 mM glucose increased $[Ca^{2+}]_i$ in the same cells. Administration of uncouplers of mitochondrial oxidative phosphorylation, 50 μM DNP and 0.3 μM FCCP, moderately elevated the basal $[Ca^{2+}]_i$ (Figure 3b and c). When this elevation was stabilized, the glucose concentration was raised to 16.7 mM; this failed to increase $[Ca^{2+}]_i$. After washing-out the uncouplers, $[Ca^{2+}]_i$ responses to 16.7 mM glucose were fully restored. Similar results were obtained with 100 μM KCN, an inhibitor of the mitochondrial respiratory chain (Figure 3d). Thus, mannoheptulose, DNP, FCCP and KCN completely inhibited the glucose-induced $[Ca^{2+}]_i$ increase in a reversible manner.

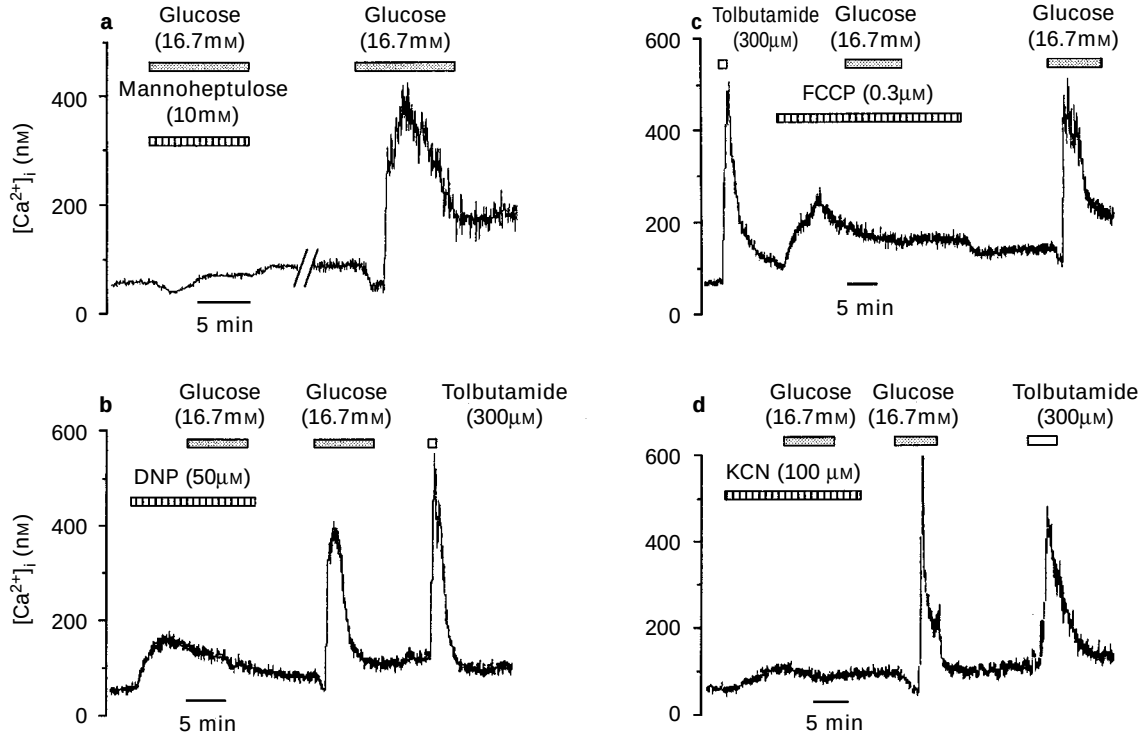


**Figure 3** Effects of inhibitors of glucokinase, mitochondrial respiratory chain and oxidative phosphorylation on glucose-induced increases in $[Ca^{2+}]_i$. Effects of 10 mM D-mannoheptulose (a), 50 μM 2,4-dinitrophenol (DNP) (b), 0.3 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (c) and 100 μM KCN (d) on 16.7 mM glucose-induced increases in $[Ca^{2+}]_i$ in single β-cells. Basal glucose concentration was 2.8 mM. $[Ca^{2+}]_i$ responses to 300 μM tolbutamide were also obtained. Results shown are representative of 6 cells in (a), 8 cells in (b), 5 cells in (c) and 6 cells in (d).

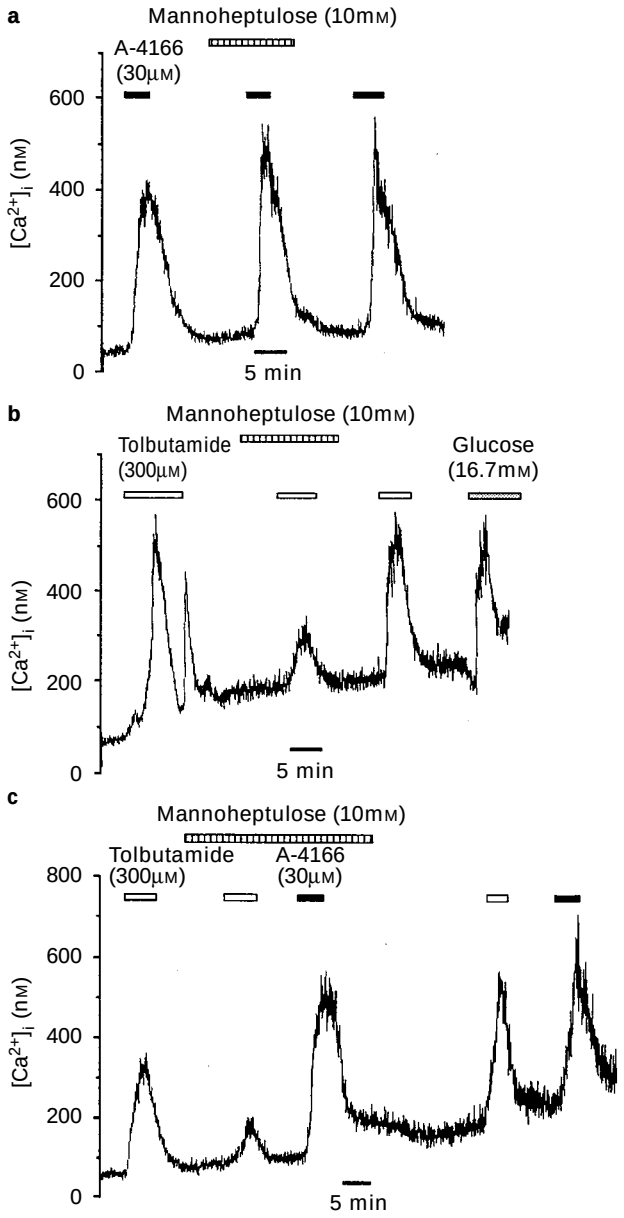


**Figure 4** Effect of mannoheptulose on the $[Ca^{2+}]_i$ responses to A-4166 and tolbutamide in single rat pancreatic $\beta$-cells. Effects of 30 $\mu$M A-4166 (a) and 300 $\mu$M tolbutamide (b) at 2.8 mM glucose in the absence and presence of 10 mM mannoheptulose, and of high glucose (16.7 mM), on $[Ca^{2+}]_i$ in single $\beta$-cells. (c) Comparative effects of 30 $\mu$M A-4166 and 300 $\mu$M tolbutamide on $[Ca^{2+}]_i$ in the same single $\beta$-cell in the absence and presence of 10 mM mannoheptulose. Note that mannoheptulose inhibited tolbutamide-induced but not A-4166-induced responses, and that tolbutamide-induced responses were fully restored upon washing-out mannoheptulose. Similar results were obtained in 9 out of 10 cells in (a) and 4 out of 10 cells in (b). The tracing in (c) represents 2 similar experiments.

### *Effects of an inhibitor of glucokinase on A-4166- and tolbutamide-induced increases in $[Ca^{2+}]_i$*

In the presence of D-mannoheptulose 10 mM, the $[Ca^{2+}]_i$ increase in response to A-4166 at the maximally effective concentration (30 $\mu$M) was not altered (Figure 4a). In contrast, the $[Ca^{2+}]_i$ response to tolbutamide at the equipotent concentration (300 $\mu$M) was either substantially suppressed in amplitude (Figure 4b) or abolished by mannoheptulose. After washing-out the inhibitor, the $[Ca^{2+}]_i$ response to tolbutamide was restored and a response to high glucose was also obtained, showing that the inhibitory effect of

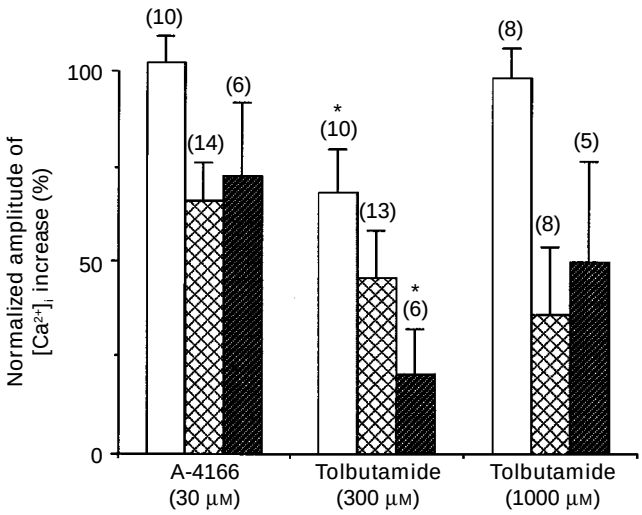


**Figure 5** Amplitudes of $[Ca^{2+}]_i$ responses to 30 $\mu$M A-4166, 300 $\mu$M and 1000 $\mu$M tolbutamide under various conditions of metabolic inhibition. Effects of A-4166 and tolbutamide at equipotent concentrations (30 $\mu$M and 300 $\mu$M, respectively), and of tolbutamide at 3.3 times the equipotent concentration (1000 $\mu$M) on $[Ca^{2+}]_i$ in single $\beta$-cells under various conditions of metabolic inhibition are summarized. Normalized amplitudes of $[Ca^{2+}]$i increase are expressed as a percentage. Procedure of normalization is described in Methods section: White columns, 10 mM mannoheptulose; cross-hatched columns, 50 $\mu$M DNP; hatched columns, 100 $\mu$M KCN. Each column represents mean $\pm$ s.e.mean for the number of cells examined, as indicated in parentheses. *Significantly different ($P<0.05$) from data with 30 $\mu$M A-4166 in corresponding condition.

mannoheptulose was quickly reversible (Figure 4b). The different susceptibility to mannoheptulose observed between the A-4166 and tolbutamide-induced responses was confirmed in a more definitive way: the $[Ca^{2+}]_i$ response to A-4166 was not affected, but that to tolbutamide was markedly attenuated in the same cell (Figure 4c). When averaged among individual $\beta$-cells, the mean amplitude of the $[Ca^{2+}]_i$ response to 30 $\mu$M A-4166 was little altered by mannoheptulose, whereas that to 300 $\mu$M tolbutamide was severely reduced (Figure 5). The amplitude distribution of $[Ca^{2+}]_i$ responses to 300 $\mu$M tolbutamide was reduced by mannoheptulose, while that to 30 $\mu$M A-4166 remained unchanged (Table 1).

### *Effects of mitochondrial uncouplers on A-4166- and tolbutamide-induced increases in $[Ca^{2+}]_i$*

At basal glucose (2.8 mM), administration of DNP, an uncoupler of mitochondrial oxidative phosphorylation, induced a moderate elevation of $[Ca^{2+}]_i$. When this change was stabilized, the effects of A-4166 and tolbutamide on $[Ca^{2+}]_i$ were examined. In the presence of DNP 50 $\mu$M, the $[Ca^{2+}]_i$ response to 30 $\mu$M A-4166 was either unaltered or suppressed (Figure 6a and Table 1). The $[Ca^{2+}]_i$ response to 300 $\mu$M tolbutamide was more severely inhibited by DNP (Table 1). The preferential inhibition of the tolbutamide-induced response is more clearly seen in the single $\beta$-cell shown in Figure 6c. The $[Ca^{2+}]_i$ response to tolbutamide even at 1000 $\mu$M, 3.3 times the equipotent concentration, was markedly inhibited by DNP (Figure 6b). The normalized amplitude of the $[Ca^{2+}]_i$ response to A-4166 was decreased but to a lesser extent than that of the response to tolbutamide at both the equipotent and 3.3 fold higher concentrations (Table 1 and Figure 5). Furthermore, another uncoupler, FCCP (0.3 $\mu$M), also suppressed the A-4166-induced response to a lesser extent than the tolbutamide-induced response (Figure 6d). In addition, upon washing-out DNP and FCCP, the responses were fully restored.

### *Effects of a mitochondrial respiratory chain blocker on A-4166- and tolbutamide-induced increases in $[Ca^{2+}]_i$*

At basal glucose (2.8 mM), the administration of 100 μM KCN, a mitochondria respiratory chain inhibitor, induced a moderate elevation of $[Ca^{2+}]_i$ in some cells. When this change was stabilized, A-4166 and tolbutamide were administered. In the presence of KCN 100 μM, the $[Ca^{2+}]_i$ responses to 30 μM A-4166 were either little altered or mildly suppressed, while those to tolbutamide 300 μM and 1000 μM were markedly inhibited in a reversible manner (Figure 7). Thus, in the presence of KCN, tolbutamide at the equipotent and 3.3 times more potent concentration was less effective than A-4166 (Table 1 and Figure 5).

### *Effects of a mitochondrial uncoupler on A-4166- and gliclazide-induced increases in $[Ca^{2+}]_i$*

Gliclazide, a second generation sulphonylurea drug, increased $[Ca^{2+}]_i$ in a concentration-related manner; the concentration inducing maximal $[Ca^{2+}]_i$ response was 30 μM for both gliclazide and A-4166 (Figure 2) (Fujitani, S., unpublished data). In the presence of DNP, the $[Ca^{2+}]_i$ response to 30 μM gliclazide was markedly inhibited in a reversible

**Table 1** Distribution of the amplitude of $[Ca^{2+}]_i$ responses to A-4166 and tolbutamide under various conditions of metabolic inhibition in β-cells

| *Condition* | *Stimulus* | *Cells with normalized response amplitude (%) of* ≤33.3[a] | 33.3–66.7 | >66.7 |
|---|---|---|---|---|
| Mannoheptulose (10 mM) | | | | |
| | A-4166 (30 μM) | 0 | 0 | 10 |
| | Tolbutamide (300 μM) | 2 | 3 | 5 |
| | Tolbutamide (1000 μM) | 0 | 1 | 7 |
| DNP (50 μM) | | | | |
| | A-4166 (30 μM) | 3 (1) | 4 | 7 |
| | Tolbutamide (300 μM) | 7 (3) | 2 | 4 |
| | Tolbutamide (1000 μM) | 6 (4) | 0 | 2 |
| KCN (100 μM) | | | | |
| | A-4166 (30 μM) | 0 | 4 | 2 |
| | Tolbutamide (300 μM) | 5 (3) | 1 | 0 |
| | Tolbutamide (1000 μM) | 3 (2) | 0 | 2 |

[a]The ≤33.3% group includes the cells in which $[Ca^{2+}]_i$ responses were abolished, the number of which is indicated in parentheses.

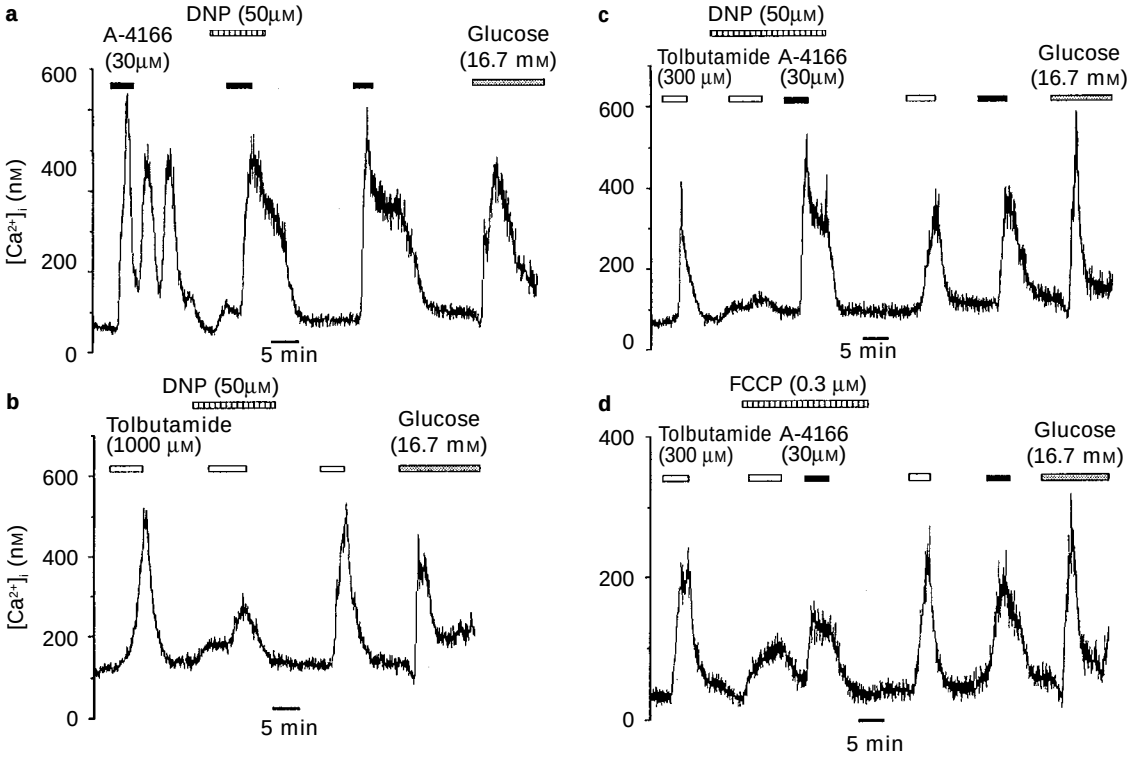


**Figure 6** Effect of uncouplers, DNP and FCCP, on the $[Ca^{2+}]_i$ responses to A-4166 and tolbutamide in single rat pancreatic β-cells. (a) Effects of 30 μM A-4166 in the absence and presence of 50 μM DNP, and of high glucose (16.7 mM), on $[Ca^{2+}]_i$ in a single β-cell. (b). Effects of tolbutamide 1000 μM, a supermaximal concentration, in the absence and presence of 50 μM DNP, and of high glucose (16.7 mM), on $[Ca^{2+}]_i$ in a single β-cell. Comparative effects of 30 μM A-4166 and 300 μM tolbutamide on $[Ca^{2+}]_i$ in the same single β-cell in the absence and presence of 50 μM DNP (c) or 0.3 μM FCCP (d). Note that DNP and FCCP inhibited tolbutamide-induced responses to a larger extent than A-4166-induced responses and that the responses were fully restored upon washing-out the inhibitors. Responses to high glucose were obtained at the end of recordings. Similar results were obtained in 8 out of 14 cells in (a) and 6 out of 8 cells in (b). The tracings in (c) and (d) represent 3 similar experiments.

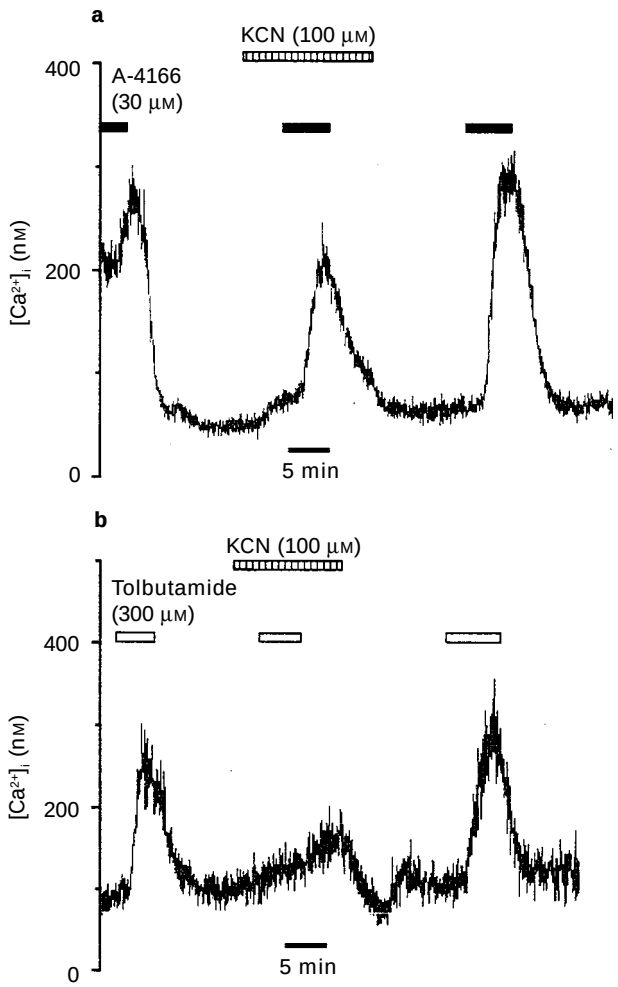


**Figure 7** Effect of respiratory inhibitor KCN on the $[Ca^{2+}]_i$ responses to A-4166 and tolbutamide in single rat pancreatic $\beta$-cells. Effects of 30 $\mu$M A-4166 (a) and 300 $\mu$M tolbutamide (b) in the absence and presence of 100 $\mu$M KCN on $[Ca^{2+}]_i$ in single $\beta$-cells. Similar results were obtained in 4 out of 6 cells in (a) and 4 out of 6 cells in (b).

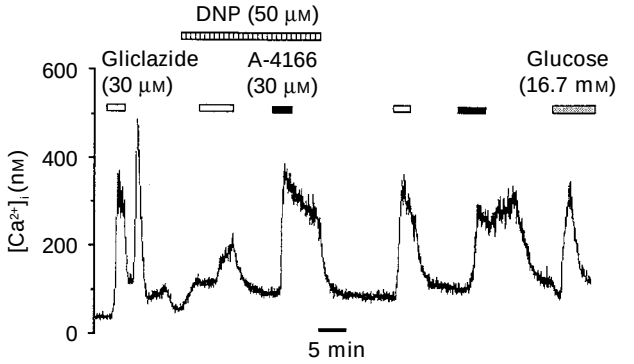


**Figure 8** Effect of gliclazide on $[Ca^{2+}]_i$ and its inhibition by DNP, an uncoupler, in single rat pancreatic $\beta$-cells. Comparative effects of 30 $\mu$M A-4166 and 30 $\mu$M gliclazide on $[Ca^{2+}]_i$ in the same single $\beta$-cell in the absence and presence of 50 $\mu$M DNP. Note that DNP inhibited gliclazide-induced responses whereas A-4166-induced responses were unaltered, and that gliclazide-induced responses were restored upon washing-out the inhibitor. The trace represents 2 similar experiments.

manner, while that to 30 $\mu$M A-4166 was not appreciably altered (Figure 8).

## Discussion

The effects of the non-sulphonylurea A-4166 and a first generation sulphonylurea tolbutamide to increase $[Ca^{2+}]_i$ in $\beta$-cells were attenuated under conditions of metabolic inhibition. The extent of attenuation was appreciably larger for tolbutamide-induced than A-4166-induced responses when the two compounds were compared at their equipotent concentrations which were determined in $\beta$-cells under normal conditions. Even the response induced by tolbutamide at 3.3 times the equipotent concentration was attenuated to a larger extent than that induced by A-4166. A comparative study of a second generation sulphonylurea gliclazide and A-4166 yielded a virtually identical result. These results indicate that A-4166 is much more resistant to metabolic suppression than sulphonylureas in increasing $[Ca^{2+}]_i$ in $\beta$-cells, the triggering signal for insulin release (Ämmälä *et al*., 1993; Gilon *et al*., 1993; Bergsten *et al*., 1994).

It was recently proposed that non-sulphonylurea oral hypoglycaemic agents, A-4166, KAD-1299 and repaglinide, can be grouped into the meglitinide family (Malaisse, 1995), the structure of which is characterized as the non-sulphonylurea moiety of a sulphonylurea, glibenclamide. It has recently been shown that the effect of repaglinide on insulin release from mouse isolated islets is unaffected by DNP-induced metabolic stress, whereas the effects of the sulphonylureas, glibenclamide and glibornuride, were impaired (Kofod & Fuhlendorff, 1995). Taken together, an effect on $[Ca^{2+}]_i$ which is relatively insensitive to the metabolic stress in $\beta$-cells may be characteristic not only of A-4166 but more generally of the meglitinide family.

It has been shown that metabolic inhibitors reduce the intracellular ATP level in a variety of cell species, and thereby inhibit ATP-dependent cellular functions such as active transport and muscle contraction (Steen *et al*., 1993; Yamamoto *et al*., 1993; Kanaya *et al*., 1995). Therefore, the present observation, that the $[Ca^{2+}]_i$ responses to tolbutamide and A-4166 were suppressed by metabolic inhibitors, suggests that an ATP-dependent process is involved in these responses. Furthermore, the finding that the A-4166 response was suppressed to a lesser extent than the tolbutamide response suggests that A-4166 is relatively more resistant than tolbutamide to a reduced ATP content in $\beta$-cells.

It has been shown that the $[Ca^{2+}]_i$ response to A-4166 involves inhibition of $K_{ATP}$ channels (Akiyoshi *et al*., 1995), activation of L-type $Ca^{2+}$ channels and stimulation of $Ca^{2+}$ influx (Fujitani & Yada, 1994), the same mechanisms as those involved in the effects of tolbutamide and other sulphonylureas (Ashcroft & Rorsman, 1989). Thus, A-4166 and the sulphonylureas apparently share common mechanisms of action in $\beta$-cells. It has recently been shown that the $\beta$-cells $K_{ATP}$ channel is a complex of the sulphonylurea receptor and an inward rectifier K channel (Inagaki *et al*., 1995). Since two nucleotide binding sites have been demonstrated for the sulphonylurea receptor (Aguilar-Bryan *et al*., 1995), it is likely that ATP regulates the sulphonylurea receptor in its binding to sulphonylureas and/or its functional coupling to the K channel, thereby also regulating the ability of sulphonylureas to increase $[Ca^{2+}]_i$. It has been shown that the ability of tolbutamide or glibenclamide to inhibit $K_{ATP}$ channels is markedly reduced by KCN, DNP or FCCP in rat $\beta$-cells (Kakei *et al*., 1995) and cardiac myoctyes (Findlay, 1993), whereas the channel sensitivity to ATP remained unchanged, suggesting that metabolic inhibition interferes with the coupling between the sulphonylurea receptor and K channel (Kakei *et al*., 1995). Therefore, a possible interpretation of our data could be that A-4166 binds to the sulphonylurea receptor, but at a molecular site which is different from that for tolbutamide binding and is functionally less susceptible to metabolic inhibition by a reduction of ATP. Indeed, it was shown that A-4166 is 51 fold, but tolbutamide is only 18 fold, more effective when the drug

was applied to the inner side of the membrane than to the outer side (Akiyoshi *et al*., 1995). This result raises the possibility that there is an as yet unidentified, A-4166-preferential receptor which is relatively more easily accessible from the inner side, and that this receptor requires a lower level of ATP than the sulphonylurea receptor. The identity and property of the A-4166-binding site remain to be clarified.

The A-4166-induced increase in $[Ca^{2+}]_i$ was little affected by mannoheptulose, while it was significantly attenuated by DNP, FCCP and KCN. It is expected that mannoheptulose inhibits glycolysis. However, amino acid and lipid metabolism can take place to feed mitochondria, resulting in partial suppression of ATP production by mitochondria. In contrast, inhibition of the mitochondrial respiratory chain or oxidative phosphorylation by KCN or uncouplers, respectively, is expected to inhibit ATP production by mitochondria strongly. It appears, therefore, that the partially reduced level of ATP by mannoheptulose is still enough to support the action pathway of A-4166, while the very low level of ATP resulting from effects of KCN or uncouplers is inadequate for maintaining this pathway.

Our results reveal that A-4166 activates β-cells under metabolic suppression much more potently than sulphonylureas. Although the mechanisms underlying the difference between A-4166 and sulphonylureas in their sensitivity to metabolic inhibition are yet to be clarified, it is suggested that the level of ATP required for A-4166 to be effective is lower than that for sulphonylureas.

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